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Electroantennogram responses of the Mediterranean fruit fly, *Ceratitis capitata* to identified volatile constituents from calling males

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Abstract

Fifty-six compounds from the odor of 'calling', sexually mature, laboratory reared males of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) were isolated by headspace trapping on Tenax columns and identified using GC/MS techniques (69 total compounds were detected). Electroantennogram responses (EAGs) to 54 of the 56 identified compounds as well as 5 analogs were tested on both sexes. Significant differences between the sexes in their responsiveness were found in 9 of the 54 identified compounds tested. There was no correlation between the amplitude of the EAG response and the relative abundance of compound identified from headspace analysis. Of the five 'major' identified components, three elicited relatively small EAG responses, while two elicited large EAGs compared to the hexan-1-ol standard. The relative ranking of EAG responses were: methyl and ethyl hexenoates and hexanoates > C_4-C_6 esters and/or acetates > ethyl and methyl octenoates > monoterpenes > sesquiterpenes > C_2-C_5 acetates, alcohols and ketones. Behavioral bioassays on each of the five 'major' identified components as well as a blend of six of the compounds showed some degree of attractancy to virgin females which in some cases approached the response to a 'pheromonal' standard (male odors absorbed onto filter paper). These results are discussed in relationship to the insect's antennal sensitivity to putative 'pheromone' components and/or allomonal components and to other reported *C. capitata* pheromone studies.

Introduction

The existence of a 'sex pheromone' emitted by adult males of the Mediterranean fruit fly, *Ceratitis capitata* (Weidemann) (medfly), was first reported by Féron (1959, 1962), although the observation of males attracting females had been made earlier (Martelli, 1910; Back & Pemberton, 1918). Jacobson *et al.* (1973) first reported the isolation, identification and synthesis of a male produced pheromone, the major components of which they identified as methyl (E)-6-nonenoate and (E)-6-nonenol. Recently, Baker *et al.* (1985) reported on the isolation and identification of the sex pheromones from male *C. capitata*, the results of which did not agree with the earlier identification by Jacobson *et al.* (1973). Baker and coworkers identified nine components from samples obtained from extended (4–5 days) headspace aeration of laboratory reared males, of which 3,4-dihydro-2-H-pyrrole (1-pyrroline) was the key pheromonal constituent based on GC/MS analysis and behavioral bioassays. Thus,

some questions still remain as to the chemical composition of the male produced odors and/or the true identification of the 'sex pheromone(s)' in this species.

Relatively little is known about the chemoreceptive basis of olfactory perception of pheromones in *C. capitata.* Nakagawa *et al.* (1973), using antennal ablation techniques, suggested that the antennae possess receptors for olfactory reception in *C. capitata* and that these chemoreceptors play an important role in mating behavior. Electrophysiological techniques such as electroantennograms (EAGs), have been recently utilized to test olfactory reception of 'pheromones' and putative pheromonal components in a number of other tephritid species (Van Der Pers *et al.*, 1984; Robacker *et al.*, 1986; Robacker & Hart, 1987).

The purpose of this study was to further identify and confirm the various sex-specific compounds and possible putative pheromone constituents produced by calling male *C. capitata* and determine the selectivity of reception of these compounds by males and females using electroantennograms and preliminary behavioral bioassays.

Materials and methods

Insects. C. capitata pupae were obtained from a mass-reared colony located at the USDA–ARS, Tropical Fruit and Vegetable Research Laboratory, Honolulu, Hawaii. The pupae were segregated as to sex prior to emergence (Cunningham, 1966). Adult flies used for EAG studies were given sugar, water, and hydrolyzed yeast, and tested 3–5 days post-emergence. Flies that were used in the collection of headspace volatiles were four days old and given only sugar and water.

Isolation and identification of male produced odors. Headspace samples of 100 four day old virgin male or female *C. capitata* were taken between the hours of 0900 and 1300, as these flies are known to 'call' and mate primarily in the morning (Wong & Nakahara, 1978). At the start of the headspace collection period, ca. 40% of the males were everting their anal ampoule, the reported pheromone gland (Lhoste & Roche, 1960), and showing characteristic wing fanning 'calling' behavior. By the end of the 3 h trapping, no males were observed 'calling'.

Headspace sampling apparatus. The sampling chamber consisted of a 5 L round bottom flask with a resin kettle neck. The head of the flask was fitted with both a Teflon inlet tube which extended to the bottom of the flask and a 1/4 inch stainless steel Swagelok fitting for attachment of a Tenax trap. The Tenax traps were constructed from stainless steel tubing (10.2 cm lengths of 0.95 cm O.D.). Traps were loaded with ca. 0.7 g of 35/60 mesh Tenax GC packing (Alltech Associates, Deerfield, IL). This provided a porous polymer column 7 cm × 0.86 cm O.D.

Headspace sampling procedure. The sampling chamber was throughly cleaned before use (hot aqueous detergent wash, distilled water rinses, and oven drying), then extensively purged with purified (activated carbon trap) breathing quality air (435 cc/min overnight). A flow rate of 150 cc/min was then established and a baked-out, clean Tenax trap attached to the outlet fitting of the sample chamber. This 'blank' control sample was trapped for 35 min, securely capped (exit end first) and placed in a polyethylene bag until analyzed.

Four day old virgin male *C. capitata* were lightly immobilized by chilling at 4 °C and ca. 100 flies were placed into the sample chamber. An air flow rate of 460 cc/min for 30 min purged the chamber for 30 min. Then the air flow was readjusted to 150 cc/min prior to attachment of a clean Tenax trap. Three different headspace samples were sequentially collected from the same individuals starting at 0900 hr for periods of 15 min (2.25 L sample volume), 30 min (4.5 L) and 180 min (27.0 L). The chamber was cleaned and a second 'blank' collected as described above prior to a 120 min collection from ca. 100 virgin females. The capped Tenax sample traps were refrigerated until GC/MS examinations were performed 10–13 days after trapping. This trapping procedure and subsequent analysis was repeated three times, in August and September, 1985 and in July, 1986.

Headspace Capillary Gas Chromatography/Mass Spectrometry GC/MS. A Finnigan 4500 GC/MS/INCOS system (Finnigan MAT, San Jose,

CA) was used for examination of headspace samples. A lab-constructed inlet system provided for thermal desorption/backflushing of loaded Tenax traps. Desorbed headspace volatiles were trapped in a liquid nitrogen (LN) chilled spiral stainless steel capillary trap, from which the volatiles were subsequently flash transferred for GC/MS examination by removing the LN bath and heating the spiral trap with a large heat gun. A 60 m×0.32 mm I.D. DB−1 fused silica capillary column (J & W. Scientific Inc., Folsom, CA) was connected to the headspace inlet system and the exit end threaded directly to the ion source of the quadrupole mass spectrometer. A flow controller maintained the helium flow through the column (2.9 cc/min at 50 °C). The column was programmed from 0° to 230 °C at 3°/min and the spectrometer repetitively scanned from 33 to 350 m/z in a one second cycle.

Headspace component identification. Sample components were tentatively identified by mass spectrum matching with a mass spectral library collection, using the Finnigan MAT INCOS data system. In the absence of suitable reference spectra, samples of suspected components were synthesized and their mass spectra acquired. Tentative MS identifications were verified by comparison of the component's experimental Kovats Index (KI) value on the DB−1 column with that of an authentic sample. The KI reference scale is the homologous series of normal hydrocarbons.

Olfactory stimuli. Based on the above analysis, a number of test compounds were chosen for assay using the electroantennogram technique (Table 1). All compounds except for 1H-Indole and 1-pyrroline were dissolved in spectrometric grade hexane (containing Ionox antioxidant) to form 10% v/v solutions. 1H-indole was dissolved in ethanol (100%) at the same 10% v/v ratio. 1-Pyrroline was made fresh prior to each test by mixing equal amounts of N-chloropyrrolidine (30.18 mg/ml) and KOH (0.4N) in salt water to yield an approximate 1% solution of 1-pyrroline (R. Binder, personal communication). Test cartridges were made by pipetting 1 μl of each test solution onto 1×2 cm pieces of fluted glass-fiber filter paper, waiting 30 seconds to allow for evaporation of the hexane, and then inserting them into pasteur pipettes. Test cartridges were loaded just prior to each presentation to an insect antenna.

The EAG and odor delivery techniques used in this study were similar to those described previously (Light & Jang, 1987; Light *et al.*, 1988). Odor stimulation periods were one sec in duration. A minimum period of 3 min of clean air preceded and followed each test compound and was found to insure full recovery of the antennal responses. EAGs were recorded from at least five different flies of each sex for each compound tested. Controls using filter paper treated with 1 μl of hexane and a standard containing 1 μl of 1% hexan-1-ol were interspersed about every fifth compound tested. Each EAG response to a test compound was converted to a percentage value of the closest standard after subtraction of the preceding hexane control. Mean responses were compared using the nonparametric Mann-Whitney U test (Snedecor & Cochran, 1967). EAG selectivity ranking was based on the above calculated percent of standard values for each sex.

Behavioral bioassays. Behavioral bioassays were carried out in screen cages 30 cm/side, containing 20 to 40 virgin female flies which were 5 to 13 days old. Flies were separated as to sex as pupae and maintained in a separate location from males thus minimizing previous exposure to pheromonal odors. Preliminary tests on the effects of age on behavioral responses indicated no significant differences in response of 5 to 13 day old virgin females maintained under these conditions. Bioassays were carried out in the morning hours when females are known to be responsive to the male pheromone. Each of the five identified 'major' headspace volatiles (Table 1) and the identified 'intermediate' headspace volatile linalool, as well as a blend of the all six of these components were tested for attractancy. In addition, observations of behaviors characteristic of pheromone-stimulated females (Ohinata *et al.*, 1973) were made for each test. Each test consisted of placing 1 μl of a 10% solution of each compound (except 1-pyrroline) in hexane onto a 4.25 cm disc of filter paper placed on a 5 cm glass petri dish. The filter paper was folded in half and placed so that the flies could orient under the odor source as desired.

Table 1. Chemical characteristics and EAG ranking of identified headspace components from the odor of calling male *C. capitata.*

	Retention			Chemical purity %	Sample[d] source	EAG Selectivity[e] ranking		Mean % EAG Response % of Standard ± SEM	
	Ref. KI	Time (sec)	Exp. KI						
Major						Female	Male	Female	Male
1 Ethyl acetate[a]	597	410	597	99+	A	49	55	64.2± 5.5	41.2± 5.7
2 1-Pyrroline[a]	652	561	655	98	B	42	45	76.4±18.4	63.4±11.6
3 Ethyl (E)-3-octenoate[a]	1179	2211	1182	94	C	12	9	165.3±16.6	180.5±13.7
4 Geranyl acetate[a]	1360	2700	1360	86	D	31	40	107.4± 5.5	78.4± 7.8
5 (E,E)-*alpha*-Farnesene[a]	1495	3035	1490	93	E	41	53	76.6± 6.4	45.7±11.2
Intermediate									
6 Dihydro-3-methyl-2(3H)-furanone[a]	900	1380	913	98	F	59	57	13.9± 7.5	21.9± 3.9
7 Myrcene	981	1605	979	80	E	11	15	168.3± 9.2	114.7±11.1
8 Ethyl (E)-3-hexenoate	987	1632	988	88	C	3	5	267.5±22.5	237.4±36.1
9 (Z)-*beta*-Ocimene	1025	1751	1026	–	N/A				
10 (E)-*beta*-Ocimene	1037	1783	1035	87	E	21	13	130.8± 9.5	148.8± 5.9
11 Linalool[a]	1083	1922	1081	97	G	28	28	111.8± 6.9	101.9± 5.9
Minor									
12 (Ethanol)[b]	440	230		100	H	54	54	47.0±17.9	44.1±13.6
13 (Acetone)	468	239		99+	I	57	59	27.5± 9.3	21.1± 8.2
14 Propyl acetate	695	666	695	98	J	47	41	67.7±11.9	77.4±14.4
15 Pyrrole	725	786	732	99	F	50	51	64.2±19.8	46.8± 6.7
16 3-Methylbut-3-enyl acetate	862	1223	865		N/A				
17 6-Methylhept-5-en-2-one	960	1542	961	80	K	9	3	172.1±16.5	256.3±54.2
18 (a monoterpene hydrocarbon)		2264	1201						
19 (a propyl octenoate? mw = 184)		2317	1220						
20 (an Ethyl octenoate? mw = 170)		2337	1227						
21 Linalyl acetate	1240	2379	1241	72	L	36	36	99.2± 1.9	80.3± 9.8
22 (a Propyl octenoate? mw = 184)		2472	1274						
23 Methyl geranate	1301	2545	1300	41	M	43	35	75.3± 7.6	80.3± 9.8
Trace									
24 (Trimethylamine)		216							
25 Methyl acetate	508	277	508	80	N	56	58	32.8±10.6	21.9± 6.8
26 2-Methylpropanol	608	455	611	99+	O	45	42	73.1±20.4	72.7±14.4
27 Pentan-2-one	658	562	655	99	F	48	50	67.4±13.8	49.6±14.1
28 Pentan-2-ol	677	628	680	98	F	5	6	230.9±21.0	223.2±17.6
29 Ethyl propionate	692	661	693	99	A	33	46	105.5±28.9	63.1±13.4
30 Dimethyldisulfide	722	717	711		N/A				
31 2-Methylpropyl acetate	750	861	755	96	P	44	43	74.1±17.5	72.5±18.3
32 Ethyl butyrate	780	948	782	99	A	14	37	154.5±14.3	79.4±14.9
33 Butyl acetate	795	992	795	98	P	46	33	72.9±12.3	86.4± 9.2
34 Ethyl but-2-enoate	819	1075	820	93	Q	6	11	200.9±17.5	158.9±26.1
35 (2,3-Butanediol)	746/756[c]	1096	827		N/A				
36 2-Pentyl acetate	830	1121	834	99	C	7	8	200.7±18.3	195.9±11.4
37 3-Methylbutyl acetate	855	1204	859	84	J	8	7	196.8±17.4	218.4±21.8
38 2-Methylbutyl acetate	858	1213	862		N/A				
39 Heptanal	875	1257	875	98	F	17	17	148.5± 8.6	140.2±11.5
40 Nonane	900	1333	898		N/A				
41 3-Methylbut-2-enyl acetate	902	1347	902	92	E	10	10	168.9±17.1	163.7±17.2
42 Benzaldehyde	926	1405	920	99	F	55	52	44.4± 3.9	47.9± 6.9
43 Methyl (E)-2-hexenoate	941	1478	942	97	R	2	1	288.0±17.8	292.9±42.9
44 Octanal	979	1597	977	96	F	26	18	113.7±13.2	137.8±28.6
45 Ethyl hexanoate	980	1609	981	98	A	4	4	259.8±19.3	249.0±29.9
46 Hexyl acetate	994	1653	994	93	F	18	21	142.7±14.0	130.8±12.4
47 p-Cymene	1011	1685	1004	90	P	30	25	107.7± 7.3	125.6±11.5
48 *gamma*-hexalactone	1003	1696	1008	98	C	52	48	58.2± 6.8	51.6± 8.2
49 Limonene	1020	1716	1014	98	K	20	16	132.5±11.1	143.6±20.5
50 Ethyl (E)-2-Hexenoate	1020	1733	1020	99	R	1	2	294.5±25.4	277.8±35.3
51 *gamma*-Terpinene	1048	1807	1044	83	O	15	14	150.8±14.7	146.9±18.6
52 2,5-Dimethyl-3-ethylpyrazine[a]	1054	1836	1053	98	C	53	49	55.3± 7.9	51.5±10.9
53 Terpinolene	1077	1897	1073	75	S	16	12	149.5±10.6	149.8±32.3
54 Nonanal	1082	1917	1080	91	G	23	24	128.8±19.2	125.7±16.7
55 Heptyl acetate	1094	1961	1094	99	P	38	39	83.8±14.7	78.7±15.0
56 Methyl (E)-3-octenoate	1108	2001	1108	91	C	27	19	111.8±12.1	137.7±15.5
57 2-Ethylhexyl acetate	1134	2079	1136	97	J	29	31	110.9±12.0	90.3±11.5
58 (an ethyl octenoate? mw = 170)		2097	1142						

Table 1. Continued.

	Retention			Chemical purity %	Sample[d] source	EAG Selectivity[e] ranking		Mean % EAG Response % of Standard ± SEM	
	Ref. KI	Time (sec)	Exp. KI						
59 Ethyl (Z)-3-octenoate	1175	2199	1178	85	T	13	22	155.4 ± 24.3	131.0 ± 6.5
60 2-Phenylethyl acetate	1224	2333	1225	99	G	32	30	106.1 ± 11.7	94.8 ± 9.2
61 Indole		2416	1254	95	P	58	56	25.0 ± 7.3	30.3 ± 4.9
62 Nonyl acetate	1293	2526	1293	97	O	25	38	122.5 ± 13.8	79.4 ± 15.9
63 (an octenoate?)		2620	1329						
64 Neryl acetate	1342	2653	1342	81	M	39	34	82.7 ± 15.6	81.4 ± 12.7
65 Ethyl (E)-3-decenoate	1374	2745	1377	90	C	35	29	99.8 ± 7.7	98.1 ± 13.5
66 (E)-*beta*-Farnesene	1447	2916	1444	77	E	37	44	95.3 ± 13.0	65.5 ± 2.5
67 Geranyl propionate	1451	2929	1449	55	G	34	27	101.2 ± 14.9	103.9 ± 12.3
68 (a Sesquiterp. H.C. mw = 204)		3004	1449						
69 (a Sesquiterp. H.C. mw = 204)		3242							
Tested analogs									
70 Methyl octanoate				94.7	L	24	23	124.9 ± 14.1	126.9 ± 11.7
71 Ethyl octanoate				99+	F	22	26	129.8 ± 9.9	116.3 ± 7.0
72 (E)-2-hexenoic acid[a]				99+	F	19	20	140.9 ± 16.5	133.7 ± 12.8
73 2,6 Dimethyl-3-ethylpyrazine				98	C	51	32	59.5 ± 8.0	86.5 ± 12.0
74 1,4 Diaminobutane (putrescine)				99	F	40	47	81.3 ± 19.5	54.9 ± 11.7

[a] Previously identified by Baker *et al.* (1985).

[b] Tentative identifications enclosed in parentheses: see text.

[c] Optically-active isomer KI = 746: meso-isomer KI = 756.

[d] *Chemical Sources:* N/A. not available

A. Alltech, Inc.
B. Sample from R. Binder, USDA-ARS-WRCC.
C. Sample from R.A. Flath, USDA-ARS-WRCC.
D. PCR, Glidden Chemical Co.
E. Sample from R. Buttery, USDA-ARS-WRRC.
F. Aldrich Chemical Co.
G. Fritzsche, Dodge and Olcott, Inc.
H. U.S. Industrial Chemicals.
I. Baker Chemical Co.
J. Chem Supply Co.
K. Fluka Chemical Co.
L. K & K Laboratories.
M. Bedoukian Research Inc.
N. Supelco Inc.
O. anonymous file at USDA-ARS-WRRC.
P. Eastman Kodak Co.
Q. Matheson, Coleman and Bell Co.
R. Sample from J. Corse, USDA-ARS-WRRC.
S. Givaudan Corp.
T. Sample from G. Takeoka, U.C. Davis.

[e] EAG ranking was based on the mean EAG response from highest (1) to lowest (59) for each sex.

This procedure also maximized the surface area over which flies could orient in response to the odors. One μl of the 1-pyrroline compound was prepared as previously described. A control consisting of 1 μl of the hexane solvent and a standard similar to that described by Ohinata *et al.* (1973), consisting of filter paper which had been exposed to a cage of 100 to 200 sexually mature males for 24 h, was run with each test. The females were observed for 10 min with each compound and scored for attractancy according to the number of females landing in or on the filter paper or petri dish.

Results

Headspace analysis of male and female produced odors. Components identified in headspace samples from the calling male flies are listed in Table 1. Each of the three different trappings showed similar qualitative identifications of compounds for each sex. All listed compounds were found in the longest duration (180 min) male headspace sample; more abundant components were found in the shorter duration male samples as well (Fig. 1). However, many of the trace components could only be identified unequivocally in the former. A smaller number of compounds at low concentrations were found in the preliminary 'blank' headspace sample. These in-

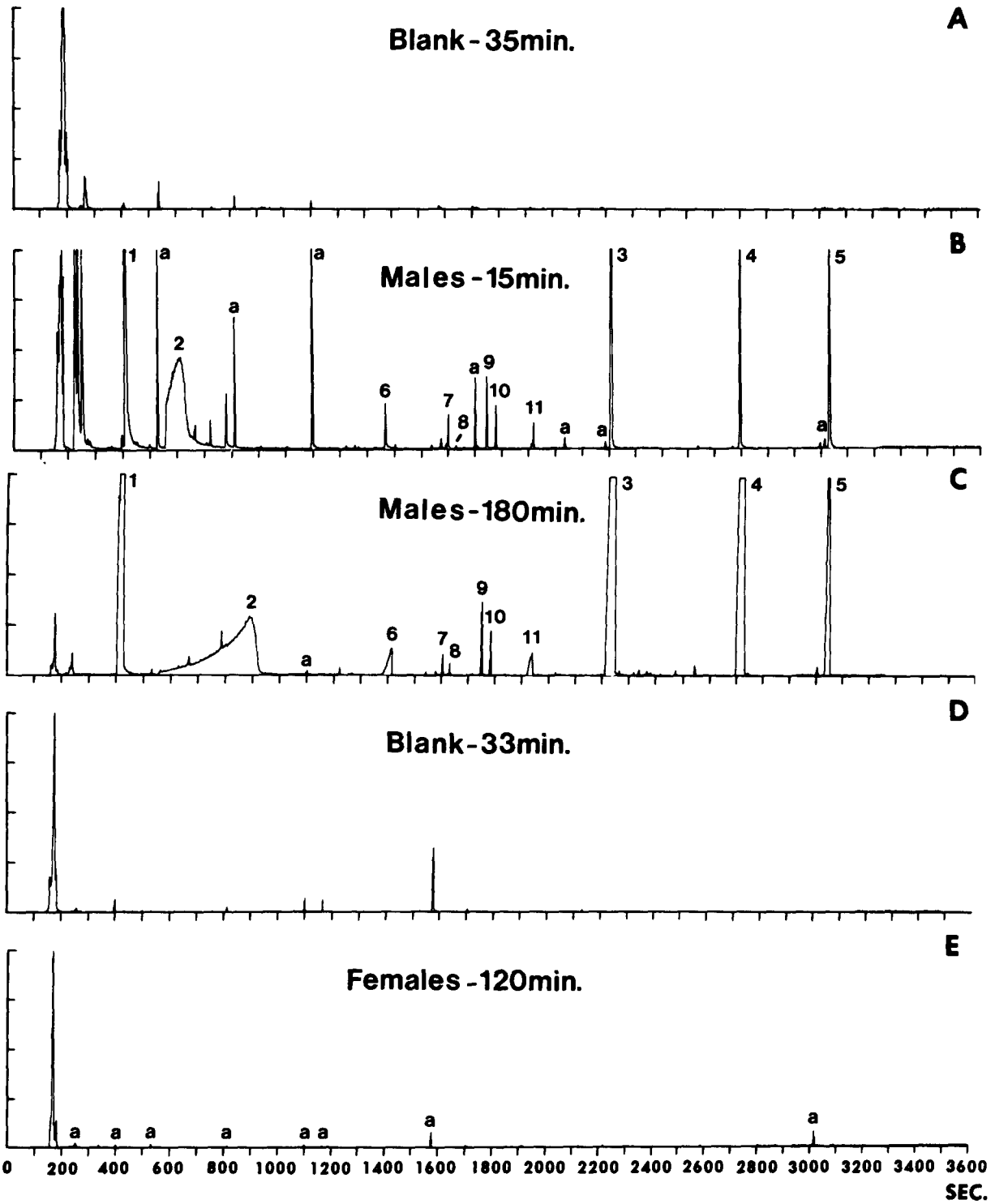

Fig. 1. Headspace GC/MS chromatograms antenuated to show principally 'major' through 'minor' components. A = System blank before introduction of males (35 min); B = Short duration male sample (15 min); C = Long duration male sample (180 min); D = System blank before introduction of females (33 min); E = Long duration female sample (120 min). Vertical attenuations were adjusted to compensate for different sampling times. Peaks representing column or system artifacts ('a'), such as the siloxanes at 1100 1702, and 2038 sec in B then appear to decrease in size in a longer sample's chromatogram (C).

cluded ethanol, 2-propanol, chloroform, benzene, 2-propyl acetate, 4-methylpentan-2-one, toluene, hexanal, 1,2,4-trimethylbenzene and column artifacts. In addition, several column artifacts were identified as siloxane peaks (see Fig. B, at 1100, 1702, and 2038 sec), the presence of which appears to be related to the water content of the individual sample. These 'blank' and column artifact compounds in the male fly samples were judged to be contaminants and were not included in Table 1, except for ethanol, which increased considerably in the headspace samples.

The male *C. capitata* headspace compounds were arbitrarily arranged in four abundance classes ('major', 'intermediate', 'minor', and 'trace'), based upon visual examination of the reconstructed gas chromatograms from individual GC/MS runs. Within abundance classes, the compounds are listed in order of increasing retention time (Table 1). A few compounds could not be unequivocally identified and are enclosed in parentheses in Table 1, as are the names of several compounds only partially characterized by mass spectral data.

In marked contrast with the male flies, very little headspace material was released by the females during the 2 h sampling period (Fig. 1E). After comparison with results from the preceeding blank run, the only compounds attributable to the presence of the females were hexanal, heptanal, nonanal, and decanal, all at trace levels in the sample.

EAG responses. A total of 54 of the 69 compounds identified from the male headspace analysis were tested on both males and females using the electroantennogram technique (Fig. 2). Significant differences between the sexes in their responsiveness to a compound were found in only 9 of the 59 compounds and analogs tested (see asterisk, Fig. 2). Of the five 'major' identified compounds tested, three of them, (ethyl acetate, 1-pyrroline and (E,E)-*alpha*-farnesene) elicited relatively low EAG responses (i.e. $<75\%$ of the 1% hexan-1-ol standard), while geranyl acetate elicited moderate EAG responses (i.e. $>75\%$ and $<150\%$) and ethyl (E)-3-octenoate elicited high EAG responses (i.e. $>150\%$) (Fig. 2). Of the five 'intermediate' compounds tested, ethyl (E)-3-hexenoate elicited the highest EAG response, myrcene, (E)-*beta*-ocimene and linalool gave moderate amplitude EAGs while dihydro-3-methyl-2(3H)-furanone elicited a very low amplitude EAG (Fig. 2). Females exhibited a significantly greater ($P<0.05$) EAG response to ethyl acetate, geranyl acetate, (E,E)-*alpha*-farnesene, and myrcene than did males.

Two of six 'minor' components and 26 of 38 'trace' compounds elicited EAG responses which equaled or exceeded the standard response level (i.e. 100%). The largest EAGs were elicited by the various methyl and ethyl hexenoates and ethyl hexanoate, followed by C_4, C_5 and C_6 ethyl esters, acetates, and/or alcohols, as well as various monoterpenes. Most of the 'minor' or 'trace' components showed no significant differences in response between the sexes. Notable exceptions were the significantly greater responses ($P<0.05$) of females than males to linalyl acetate, ethyl butyrate, nonyl acetate, and (E)-*beta*-farnesene, while only 6-methylhept-5-en-2-one elicited significantly greater EAGs in males than females.

Several other compounds were also tested, because of either their structural similarity to identified components (putrescine, 2,6-dimethyl-3-ethyl pyrazine, and methyl and ethyl octanoates) or the fact that they have been previously reported as a male-produced compound ((E)-2-hexenoic acid) from *C. capitata*. EAG responses to both the methyl and ethyl octanoates were lower than to methyl (E)-3-octenoate and ethyl (Z)-3-octenoate and significantly lower than responses to the 'major' component, ethyl (E)-3-octenoate. (E)-2-Hexenoic acid, a compound previously identified as being produced by males (Baker *et al.*, 1985) but not found in our samples, elicited an EAG response which ranked in the top one-third of the compounds tested (Table 1).

EAG ranking. In general, there did not appear to be any correlation between the relative amounts of compounds identified from the head space analysis and their potency as measured by EAGs (Table 1). Potency rankings for the compounds were for the most part similar between the sexes. Only one of the identified five 'major' components (ethyl (E)-3-octenoate) was ranked in the top half of all the compounds tested on both females and males. Four of the five 'intermediate' compounds ranked in

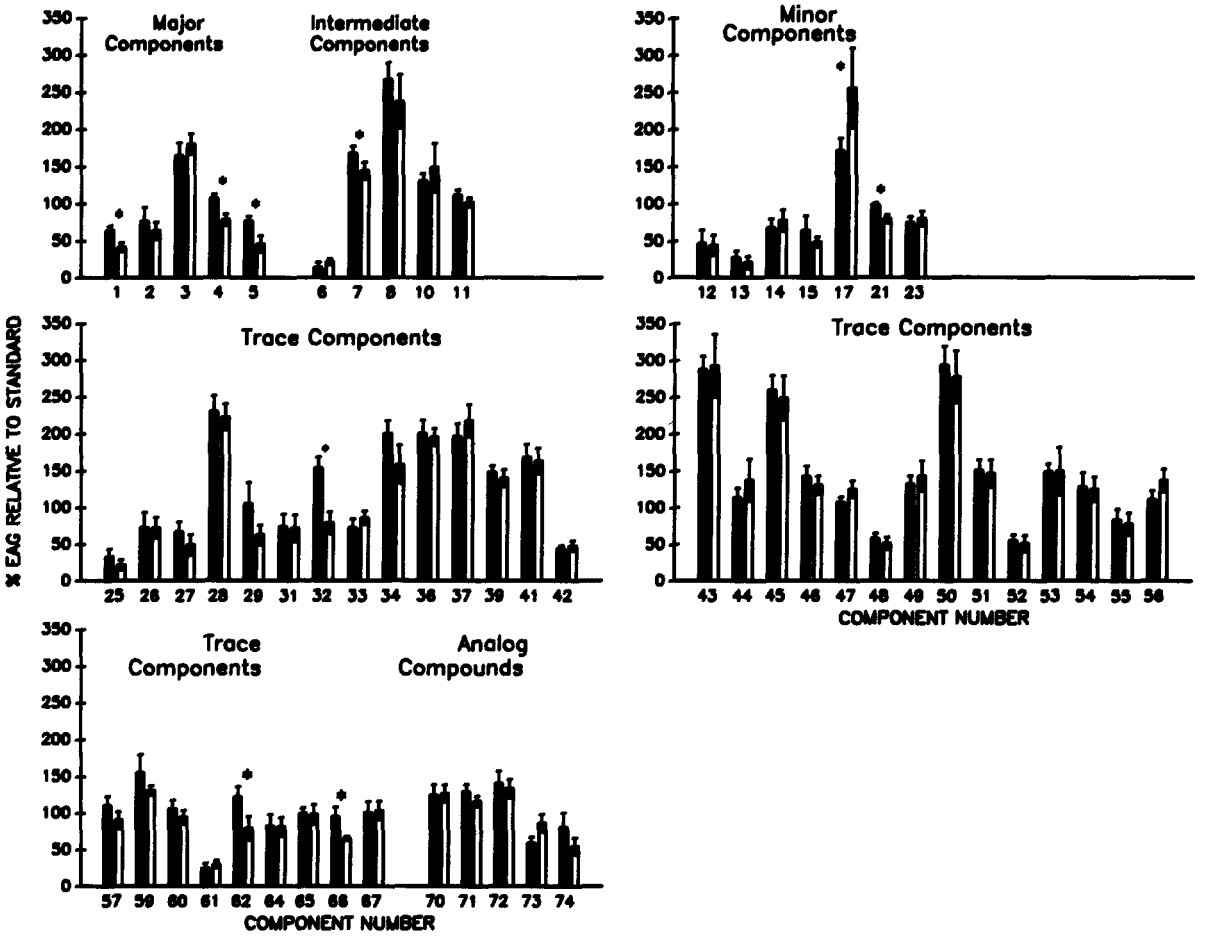

Fig. 2. Electroantennogram responses of male (Blank bars) and female (filled bars) *C. capitata* to compounds identified from headspace volatile analysis (GC/MS) of calling males ($n > 5$/sex). * Indicates significant differences between the sexes ($P < 0.05$). Compounds are arranged by their retention times in each abundance class and compound number reflects the numbered compounds listed in Table 1.

the top half of all compounds tested, with one of these (ethyl (E)-3-hexenoate) ranked in the top five for both sexes. Only one 'minor' component (6-methylhept-5-en-2-one) ranked in the top 50 percent. Eight of the ten compounds eliciting the greatest EAG response in females and seven of ten for males were identified as 'trace' components. As a class, methyl and ethyl esters elicited the greatest EAG responses followed by various acetates, monoterpenes, and alcohols. Five of the top ten compounds in the female ranking were ethyl or methyl esters (four of these being hexenoic or hexanoic acid esters) and three were acetates. Antennal specificity to structurally similar compounds within a class were evident in a number of cases. Both sexes showed a greater response to the C_6 acid (acyl) esters than compounds with a C_6 (alkyl) alcohol chain. Males were significantly more responsive to the (E) vs. (Z) configuration and unsaturated vs. saturated C_8 ethyl esters. This was not evident in the C_8 methyl esters.

Long recovery. An unusually long recovery of the EAG trace to baseline after the one sec odor stimulation was often seen for compounds of certain functional group classes. 'Long recovery' EAG recordings were defined as those in which the trace did not return to its original baseline within the field of view of the oscilloscope screen (ca. 10 sec) and were characterized by a recovery trace having a much

more gradual slope and a significantly longer time period required for the trace to return to its original baseline (Jang *et al.*, in press). Sixteen of the 59 compounds tested in this study showed 'long recovery' for a majority (>50%) of the replications performed on at least one sex. As a class, esters having an acid (acyl) chain longer than 2 carbons (i.e. non-acetates) had the greatest incidence of long recoveries (6 of 13 esters tested exhibited >50% long recovery for at least one sex) followed by the monoterpenoids and sesquiterpenes (6 of 13), and the alcohols, ketones and pyrazines (2 of 7). The hexenoates which evoked some of the most potent EAG response (Table 1) also showed the highest incidence of long recoveries.

Behavioral bioassays. Response to the standard filter paper containing the absorbed male odors was variable with percent attraction ranging form zero to 42% of the flies responding in any given test. A mean of ca. 15% of the flies overall responded to the standard (Table 2). Compared to the standard, considerable variation was also noted in the attraction of virgin females to individual compounds. All tested compounds showed a significant degree ($P < 0.05$) of attraction over the filter paper blank. Both 1-pyrroline and the six-component blend elicited mean responses of approximately 10%, while the other chemicals alone elicited responses which ranged from 3.7% (geranyl acetate) to 9.2% ((E,E)-*alpha*-farnesene) of the females attracted. The blend, in addition to eliciting a comparable degree of attraction to that of the standard, also elicited in most of the female flies behaviors characteristic of the pheromone response such as rapid orientation to the source, slow wing fanning, head butting and bobbing of the abdomen. All compounds elicited one or more of the mentioned behaviors during the course of at least one of the tests. However, the responses to individual compounds were not as obvious as to the artificial blend or to the filter paper standard and appeared to be more or less randomly distributed throughout the single compounds tested.

Discussion

Identification of male produced volatiles. Headspace samplings of male *C. capitata* over various time periods on three separate occasions resulted in the identification of 56 compounds as well as tentative or partial identification of 13 additional compounds using GC/MS techniques. The number and diversity of chemicals identified from the male odor was in sharp contrast to the virtual absence of compounds from headspace analysis of the female odor. Most of the experimental KI values are within a few units of the corresponding reference KI values determined with authentic samples. The chromatographic peak shapes of some of the components were poor. The 1-pyrroline peak was not symmetrical even in the 15 min sample and smeared badly. In such cases, KI values near the fronts of the peaks were assigned.

Baker *et al.* (1985) using aeration trapping over a 4–5 day period identified 9 compounds emitted by male *C. capitata.* All of these, with the exception of (E)-2-hexenoic acid, were also identified in the present study, including all five 'major', two 'intermediate' and one 'trace'. In the headspace samples collected in this study, 40–45% of the males present were observed (initially or throughout the sampling period) 'calling' (i.e. tilting of the abdomen and eversion of anal ampoule with concommitant rapid backward fanning of the wings) a behavior associat-

Table 2. Response of virgin female *C. capitata* to identified major headspace volatile components, singly and as a blend compared to a filter paper substrate of absorbed natural pheromone.

Compound	Number of replications	Mean percent of caged flies landing on filter paper substrate ± SEM
1. 1-pyrroline	9	11.1 ± 3.8
2. Ethyl acetate	8	5.3 ± 1.5
3. Ethyl (E)-3-octenoate	6	5.8 ± 4.0
4. Geranyl acetate	6	3.7 ± 1.2
5. (E,E)-*alpha*-farnesene	6	9.2 ± 3.5
6. Linalool	9	5.8 ± 3.7
7. Blend[a]	9	10.9 ± 4.7
8. Filter paper control	8	0.9 ± 0.7
9. Filter paper exposed to males (standard)	6	14.6 ± 7.1

[a] Blend contained a 4:1:1:1:0.7:0.4 ratio of 1-pyrroline:ethyl acetate:ethyl (E)-3-octenoate:geranyl acetate:(E,E)-*alpha*-farnesene:linalool.

ed with release of the 'sex pheromones' in both laboratory (Féron, 1959, 1962) and wild (Prokopy & Hendrichs, 1979) flies. Quantitative discrepancies in relative concentrations of the major components between our results and those of Baker and coworkers (1985) most likely occurred because of differences in methodology, especially in the duration of the collection of the respective samples, and/or the sources of flies used. Our results were qualitatively consistant with Baker *et al.* (1985) in that we could find no evidence for the presence of (E)-6-nonenol, methyl (E)-6-nonenoate, or the spectrum of saturated aliphatic acids which Jacobson *et al.* (1973) found in an aeration, cold-trapped concentrate from laboratory males.

EAG responses. The overall similarity of antennal responses of male and female *C. capitata* to male produced odors indicate that both sexes are capable of detecting a broad spectrum of the identified compounds; one or more of which most likely constitute components of the 'sex pheromone' of this species. In this study, the identified components of the male *C. capitata* odor found to be the most potent antennal stimulants were the C_6 acid esters (hexenoates and hexanoates) followed by C_4 to C_6 esters and acetates, ethyl octenoates, and monoterpenes. The precise roles of these highly stimulatory compounds on the sensory behavior of *C. capitata* and their contributions as components of a putative sex pheromone are at present not well understood.

Although we did not find a correlation between relative abundance of male odor components, antennal potency, and/or 'sex pheromone' behavior in *C. capitata*, the lack of apparent antennal sensitivity to some of the compounds (i.e. most 'major' to 'minor' components) does not imply a lack of their behavioral importance. Since EAG dosage/response studies have not yet been carried out with these compounds, we are not yet able to determine if those 'major' components having low EAG responses (e.g. 1-pyrroline, ethyl acetate, or (E,E-*alpha*-farnesene) perhaps have lower threshold values for detection than 'intermediate', 'minor' or 'trace' compounds which evoked larger EAG responses. Alternatively, 'trace' compounds eliciting large EAG responses at the concentrations tested may show lower responses at more biologically relevant doses.

EAG responses either to components of the 'sex pheromones' or to volatiles produced by male flies have been reported for at least three other tephritid species. Van der Pers *et al.* (1984) recorded EAG responses of male and female olive fruit flies (*Dacus oleae* (Gmel.)) to six 'pheromonal' and five analog compounds. They found that the major pheromone component, 1,7-dioxaspiro-(5,5)-undecane, had a lower threshold value than all other compounds tested. However, they found higher absolute EAG responses to nonanal and an analog (1-octanol) than for the major pheromone component. In contrast, Robacker *et al.* (1986) found that (S,S)-(−)-epianastrephin, the major pheromone component of the Mexican fruit fly, evoked larger EAG responses than any of the other three identified lactone pheromone components (Battiste *et al.* 1983; Stokes *et al.* 1983) either alone or blended with the other three. Robacker and Hart (1987) working with the Caribbean fruit fly, reported both sexes responded similarly in EAG studies to a majority of the tested male produced chemicals, both singularly and in combination. Trimedlure (*tert*-butyl-4 (and-5)-chloro-*trans*-2-methylcyclohexane-1-carboxylate), a known potent male attractant for *C. capitata* also elicits relatively low EAG responses (i.e. <70% of standard responses) over a broad concentration range but has a lower threshold and higher EAG response to its most active isomer (Jang *et al.*, in press).

The 'trace' component 2,5-dimethyl-3-ethylpyrazine, which elicited a relatively low EAG response in this study was also identified by Baker *et al.* (1985) from headspace volatile trappings of male *C. capitata.* Pyrazine-type compounds have been reported as pheromone components of other tephritid fruit flies including the melon fly, *Dacus cucurbitae* Coquillett (trimethylpyrazine, tetramethylpyrazine, and methylpyrazine; Baker *et al.*, 1982), and the papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker (2-methyl-6-vinylpyrazine; Chuman *et al.*, 1987). Both 2,5-dimethyl-3-ethylpyrazine and trimethylpyrazine have also been identified from headspace analysis of the oriental fruit fly, *Dacus dorsalis Hendel (Flath et al.,* unpublished). This occurrence of pyrazines in at least four tephritid fruit fly species and the apparent species

specific differences in pyrazine composition may suggest a functional role for these compounds as constituents of their overall 'pheromone' blends, or perhaps as constituents of interspecific allomonal communication among the species.

Our finding of the virtual lack of detectable volatiles emanating from laboratory-reared female *C. capitata* is in agreement with results reported by Baker *et al.* (1985) and perhaps suggests that the large number of compounds identified from males may play a functional role in either female attraction and subsequent courtship (sex pheromones) or male induced lekking, competition, or aggregation behaviors (Arita & Kaneshiro, 1985). Virgin females were attracted to both the standard (a filter paper exposed to males) and the artificial blend of the five 'major' and one intermediate (linalool) identified components from the headspace volatiles. Ohinata *et al.* (1973) reported that using a similar filter paper standard in their sex pheromone bioassays resulted in attraction of ca. 50% of the virgin female *C. capitata* released. Although the standard response in the present study was low (ca. 15%), possibly due in part to fewer numbers of active males creating the standard, the characteristic behaviors associated with the presence of sex pheromone, (e.g. wing fanning, head butting, rapid orientation to the source and abdomen bobbing) were observed in virgin females, especially in response to the six-component blend. 1-Pyrroline, the compound found by Baker *et al.* (1985) to be extremely active in Y-tube attractancy assays, also showed some efficacy in our cage bioassay (Table 2). Each of the other compounds tested singly also elicited some low level response, contrary to the study of Baker *et al.* (1985) who tested some of the same compounds and found no activity with individual components. These differences most likely result from differences in methodology.

Males and females evoked very similar EAG responses to a majority (49) of the 59 compounds tested in this study. This similarity in EAG response of the sexes has been reported for many of the tephritid species studied to date for both host fruit and plant volatiles (Fein *et al.* 1982; Light & Jang, 1987; Light *et al.*, 1988) and identified pheromone components (Van Der Pers *et al.*, 1984; Robacker *et al.*, 1986; Robacker & Hart, 1987). On the other hand, differential EAG responsiveness between the sexes in various insect species has been utilized extensively to screen for putative sex pheromone components that are then tested in behavioral bioassays, such as the studies of Nishino and coworkers on the American cockroach, *Periplaneta americana* (Nishino & Kimura, 1981; Nishino & Takayanagi, 1981). We found 9 of the 54 identified compounds tested evoked EAG responses that had significant differences in magnitude between the sexes, with females having greater response amplitudes than males to all but one of the nine compounds. These nine compounds were among the most abundant components of the male odor bouquet, containing three of the five 'major' components, one of the five 'intermediate' components and two of the seven 'minor' components as opposed to only three of the 47 'trace' components tested. The three 'major' components (ethyl acetate, geranyl acetate, and (E,E)-*alpha* farnesene) also elicited some behavioral responses in virgin females.

The observed phenomena which we refer to as 'long recovery' may be the result of either differences in the relative affinity of the molecules with the acceptors, or differential 'inactivation' of the ligand–molecule acceptor sites. 'Long recovery' has also been reported to be evoked by the attractive isomers of the male *C. capitata* attractant trimedlure, while 'normal' recoveries were observed following stimulations by the non-attractive components (Jang *et al.*, in press). Thus, differences in size and shape of the EAG responses may suggest differences in overall size or affinity of receptor populations on the antenna for different classes of compounds. How these suggested receptor endowments and their physiology correlate with higher centers of integration and ultimately behaviors are not yet fully understood. However, behavioral specificity, at least with regards to the sex pheromone in *C. capitata* may be dependent on factors other than the mere magnitude of receptor populations for the various components. Flight-tunnel behavioral bioassays are in progress to more fully determine the various semiochemical functions of these identified male-specific odors of *C. capitata*. In addition, single cell recordings will be undertaken to study the sensitivity and specificity of specific receptors to these molecules.

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Résumé

Electroantennogrammes de Ceratitis capitata *(Dipt.: Tephritidae) en présence de composés volatiles isolés et identifiés, produits par des mâles en appel*

Cinquante-six composés de l'odeur de mâles de *C. capitata* Weidemann, élevés en laboratoire, sexuellement mûrs et en appel, ont été isolés par piégeage sur colonnes tenax et identifiés par la technique GC/MS (69 composés avaient été détectés en tout). Les électroantennogrammes (EAGs) ont été examinés chez les deux sexes pour 54 des 56 composés identifiés et 5 de leurs analogues. Des différences significatives entre les sexes ont été observées pour 9 des 54 composés identifiés. Il n'y avait pas de corrélation entre l'ampleur de l'EAG et l'abondance relative du composé lors de son isolement. Pour les 5 principaux composés identifiés, 3 ont induit des EAGs relativement faibles, tandis que 2 étaient importants, par comparaison avec l'Hexane-1-ol utilisé comme témoin. Le classement relatif des EAG a été: hexénoates et hexanoates d'éthyl et de méthyl $\geq C_4-C_6$ esters et/ou acétates $\geq$ octénoates d'éthyl ou de méthyl $\geq$ monoterpènes $\geq$ sesquiterpènes $\geq C_2-C_5$ acétates, alcools et kétones. Les expériences de comportement avec chacun des 5 composés principaux identifiés, comme avec des mélanges de 6 composés ont mis en évidence une attraction des femelles vierges qui dans quelques cas avoisine la réponse à la phéromone témoin (odeur du mâle absorbée sur papier filtre). Ces résultats sont discutés en fonction de la sensibilité de l'antenne d'insexte aux composés supposés de la phéromone et aux composés allomonaux, et en fonction des autres études connues sur les phéromones de *C. capitata*.

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